

# Control of Pigment Biosynthesis Genes during Petal Development

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## INTRODUCTION

Sexual reproduction in flowering plants depends on the evocation of flowers, which, in general, consist of four whorls of organs: sepals, petals, anthers, and pistil. Sepals and petals can be regarded as initially protective organs. During the first stage of floral development, meiosis occurs in the anthers and the pistil while these organs are still enclosed by the sepals. Petals, the organs of the second whorl of the floral meristem, start developing toward the end of meiosis. Their growth coincides with the formation of the anther filaments and the stem of the pistil. Finally, during flower maturation, the petals unfold to perform a second function in plants pollinated by insects or birds: their color serves as a flash signal to attract pollinators, and their structure serves as a landing place. Here, we will describe one aspect of petal development, the coordinated expression of the color genes whose combined activities produce the visual signals that attract pollinators to flowers.

## PETAL DEVELOPMENT

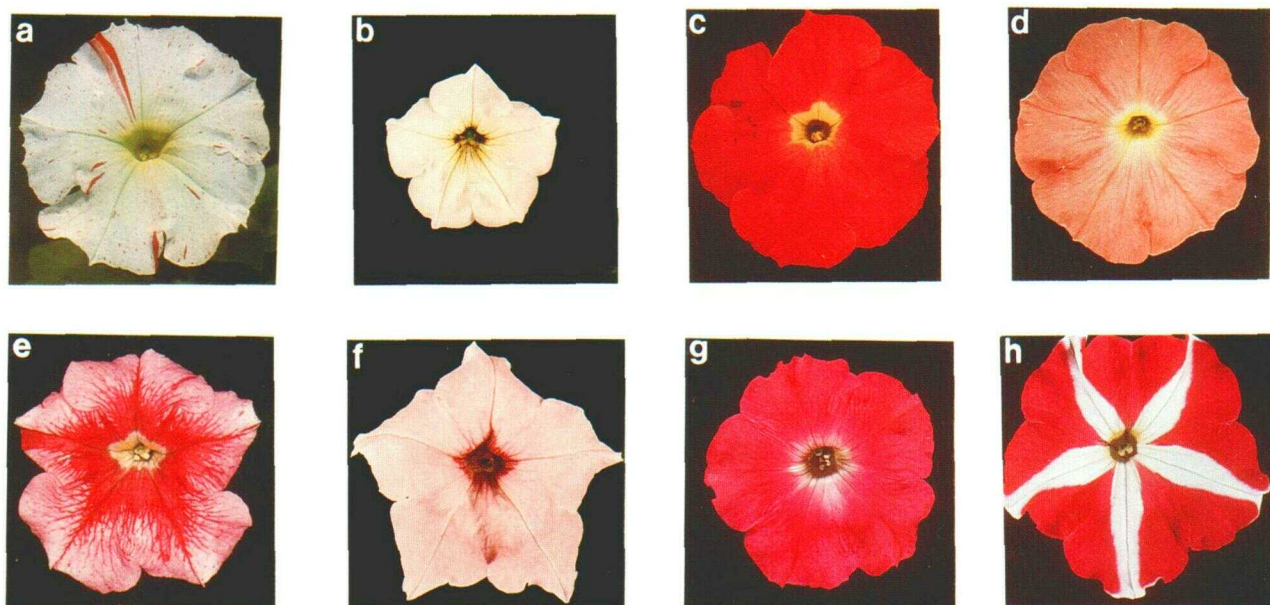
In species such as tobacco and petunia, the corolla consists of five partly or completely fused petals and is divided into two distinct regions, the tube and the limb. This anatomical differentiation is often accompanied by a differential coloration pattern, as shown in Figure 1B, in which the petunia flower tube is colored but the limb is acyanic, although other patterns of pigmentation, such as veination and star forms, may be observed in flowers (e.g., as shown in Figures 1E and 1H). The links that exist between the complex of genes that govern floral morphogenesis and the events that lead to the local induction of color formation are now beginning to be unraveled.

Petals develop from petal primordia in the second whorl of the floral meristem, which form by periclinal divisions in the lower cell layers. Subsequent vertical growth requires the activity of subapical initial cells, and lateral growth requires the activity of submarginal initials. Flowers with corollas formed from fused petals initiate separate petal primordia. The process

by which the petal primordia subsequently fuse to form the corolla varies in different species. In *Antirrhinum*, the tissue at the base of the stamens on the abaxial side and in the interprimordial region between the petal primordia starts to divide to form a collar of tissue in combination with the petal primordia (Awasthi et al., 1984). The corolla tube therefore forms from zonal growth of the petal primordia, the interprimordial region, and the abaxial base of the stamens. This effectively unites the stamens with the corolla so that they are epipetalous. The cells at the base of the tube mature early. Only the upper part of the zonal meristem, above the point at which the petals and stamens join, continues to divide to form the upper part of the corolla tube. Therefore, the cells of the lower and upper parts of the corolla tube can be viewed as morphologically distinct.

Anthocyanins are formed primarily or exclusively in the epidermal cells of flowers, and an understanding of the development of the epidermis in petals may facilitate an understanding of floral patterning. Meristematic activity in tobacco petals is located at their margins (Drews et al., 1992). Analysis of reversion patterns of unstable color genes in petunia has led to the idea that the epidermis of the petal limb is derived from two independent rings of meristematic cells located at the rim of the developing corolla. One of these rings is the source of cells for the upper or inner epidermis, and the other is the source of cells for the lower or outer epidermis. Two observations have led to this idea. First, colored sectors that do not reach the margin of the corolla (internal sectors) are usually oval; they terminate in a single cell at both the proximal and distal ends (Figure 1A). Internal sectors would thus appear to arise from a limited number of cell divisions that occur after the cells have separated from the marginal meristem. Sectors that extend to the margin of the corolla are, without exception, triangular, with their bases at the margin (Figure 1A). These triangular sectors fall into two classes: they are either confined to the upper or lower epidermis or they "go over the edge." In the latter case, the sectors on the upper and lower epidermis usually diverge at their proximal ends. At the distal end, they invariably meet at the same cell on the margin of the corolla. This can be explained by assuming that, although usually separate, cells from the upper meristematic ring may

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**Figure 1.** Variation in Pattern and Intensity of Anthocyanin Production in Petunia Flowers.

- (A) Flower showing red sectors on an acyanic background due to an unstable *an1* allele. Sectors can be used to map development of the epidermis.
- (B) Phenotype of Mitchell line, showing an acyanic limb. The tube accumulates some anthocyanin and appears darker.
- (C) Fully colored line showing anthocyanin pigment in the limb.
- (D) Pale self-colored allele (compare with [C]) resulting from imprecise transposon excision from the *an1* locus, illustrating that not all mutations give rise to pigmentation patterns.
- (E) Pattern of pigmentation resulting from mutation of the *an12* locus. Pigmentation is concentrated over the veins. This allele is unstable and shows reversion to restore *An12* activity as large self-colored sectors (for example, on the central left-hand petal).
- (F) Pigmentation pattern resulting from the separate fields of activity of the *Ht1* and *Ht2* genes within the petunia flower. In this flower, *Ht1*, which governs F3'H activity in the lobes, is nonfunctional. Dihydrokaempferol therefore accumulates, but it cannot be converted to pelargonidin due to the substrate specificity of DFR in petunia. *Ht2*, which governs F3'H activity in the tube, is functional in this line. The tube therefore is darkly pigmented due to the formation of cyanidin.
- (G) Full red line of petunia to contrast with (H).
- (H) "Red Star" phenotype showing acyanic sectors in a colored background.

invade the lower ring or vice versa, perhaps as a result of shifting mechanical forces or local changes in rates or patterns of cell division. This arrangement of epidermal cells means that the cells at the margin of the limb are developmentally younger than internal cells.

It seems likely that this is a general scheme for epidermal development in petals because many species with unstable color genes show similar types of sectors, including *Antirrhinum*, *zinnia*, *morning glory*, and *Mirabilis* (Spitters et al., 1975; Epperson and Clegg, 1987; Fincham, 1987; Smith et al., 1988). The cells of the inner epidermis of the limb or lobe also develop a specialized conical form compared to the normal flattened shape of epidermal cells. These conical cells contain the highest concentrations of anthocyanins, and they reflect incident light to enhance the impact of their pigment as a flash signal (Kay et al., 1981).

Cell division in developing petals ceases at ~5 to 6 days before anthesis. Increases in somatic reversion frequencies of unstable color genes can be induced by environmental changes, but during the last 5 to 6 days before maturation, no such increases can be induced (Bianchi et al., 1978; Doodeman et al., 1985), indicating that cell division has ceased. At this point, the flower is only 40% of its final length. The further growth and unfolding of the flower bud, which shapes the final flower, is, therefore, primarily a result of differential cell elongation. Virtually nothing is known about this part of the developmental mechanism, which leads to complex forms such as those seen in the *Antirrhinum* flower. Although some of the patterning of pigmentation in flowers reflects morphological boundaries, the induction of pigment synthesis occurs during the period of cell expansion, after the establishment of many of the boundaries in petal form (Coen et al., 1986).

## FLOWER COLOR SYNTHESIS

Under natural conditions, flowers attract pollinators with their color, which is therefore an important component of their development. The major flower pigments are flavonoids, particularly anthocyanidin glycosides, which confer colors ranging from orange to violet. Other flavonoids, the aurones and chalcones, give rise to yellow colors in flowers, although orange, brown, and yellow colors also arise from the production of unrelated carotenoids or mixtures of these with flavonoids.

Synthesis of anthocyanins requires the concerted action of at least six committed biosynthetic enzymes, and in many species more are involved. These enzymatic steps are listed in Table 1. The isolation of the structural genes involved in flavonoid biosynthesis has been achieved by a combination of genetic, biochemical, and molecular approaches (for example, Wienand et al., 1982; Reimold et al., 1983; Fedoroff et al., 1984; van Tunen et al., 1988; Martin et al., 1991). The majority of the genes encoding the enzymes catalyzing the biosynthetic steps have been cloned, allowing the study of their expression during flower development.

## PIGMENT SYNTHESIS IS INDUCED DURING PETAL DEVELOPMENT

The formation of anthocyanins is induced during petal development primarily as a result of increased synthesis of the biosynthetic enzymes. In every case that has been examined, the steady state levels of the transcripts of each biosynthetic gene increase during the formation of colored flowers, especially during the period of petal cell expansion, as a result of increased gene transcription (van Tunen et al., 1988; Beld et al., 1989; Koes et al., 1989; Jackson et al., 1992).

In maize, pigmentation of the aleurone cell layer in the kernels involves the simultaneous induction of all the biosynthetic genes. This multiple transcriptional activation is coordinated by two transcription factors, *R* and *C1*, which act together to induce all the committed biosynthetic genes (Dooner, 1983; Cone et al., 1986; Paz-Ares et al., 1986; Chandler et al., 1989; Ludwig et al., 1989). In flowers of dicots, by contrast, there does not appear to be a single induction mechanism for all of the biosynthetic genes; rather, there is evidence for discrete control of different biosynthetic genes (Almeida et al., 1989; Martin

**Table 1.** Enzymes Required for Anthocyanin Biosynthesis

Enzyme	Abbreviation	Enzymatic Activity	Comments
Chalcone synthase	CHS	Condensation of 4-coumaroyl CoA and malonyl CoA to form naringenin	Required for the synthesis of aurones, flavanones, flavones, flavonols, and anthocyanins
Chalcone isomerase	CHI	Stereospecific ring closure of chalcone to form naringenin (flavanone)	There is also some nonenzymatic conversion of this step, which is required for the synthesis of flavanones, flavonols, and anthocyanins
Flavanone 3-hydroxylase	F3H	Hydroxylation of flavanones (naringenin or eriodictyol) on the 3 position to form dihydroflavonols	Required for synthesis of flavonols and anthocyanidins
Dihydroflavonol 3'-hydroxylase	F3'H	Hydroxylation on the 3' position	Enables production of red/magenta cyanidin
Dihydroflavonol 3',5'-hydroxylase	F3'5'H	Hydroxylation on the 3' and 5' positions	Enables production of blue/purple delphinidin
Dihydroflavonol 4-reductase	DFR	Reduction of dihydroflavonols to leucoanthocyanidins	Required for synthesis of anthocyanidins
Anthocyanidin synthase	AS	Encodes a dioxygenase	Conversion of leucoanthocyanidins to anthocyanidins not yet fully elucidated. A second step may be required, possibly a dehydratase (Heller and Forkmann, 1988)
UDP-glucose flavonol 3-O-glucosyl transferase	UGFT	Glycosylation of anthocyanidins and flavonols on the 3 position	
Rhamnosyl transferase	RT	Rhamnosyl addition to glucose to form rutinoside	

et al., 1991). The evidence for separate regulatory mechanisms is twofold. First, detailed time-course analysis of steady state transcript levels for six of the biosynthetic genes in developing Antirrhinum flowers (Jackson et al., 1992) shows that the genes encoding the first two steps of the pathway, chalcone synthase (CHS) and chalcone isomerase (CHI) (early biosynthetic genes [EBGs]) have different expression profiles than the genes encoding flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (AS), and UDP-glucose flavonol 3-O-glucosyl transferase (UGT) (late biosynthetic genes [LBGs]). The CHS and CHI expression profiles also differ from each other. These differences imply that whereas some factors involved in transcriptional activation may control expression of all of the biosynthetic genes, other factors must be specific for subsets of biosynthetic genes or individual biosynthetic genes. Similar results have been found in Arabidopsis pigmentation induced by light, in which differences between the time courses of expression of the EBGs CHS and CHI and the LBG DFR can be observed (Kubasek et al., 1992). In petunia flowers, detailed time courses have shown that steady state levels of the EBGs CHS and CHI peak earlier than those of the LBGs (Weiss et al., 1993).

The second line of evidence for discrete regulation of sections of the pathway comes from the study of the effects of mutations in the regulatory genes. In Antirrhinum, the *Delila* (*Del*) gene product is required for anthocyanin biosynthesis in the flower tube (Figures 2A and 2B). In the absence of a functional *Del* product, there is no expression of the LBGs in the flower tube, although the EBGs are expressed in the tube (Almeida et al., 1989; Martin et al., 1991). This indicates that although *Del* is an activator of the LBGs in tubes, it is not necessary for activation of the EBGs in tubes. Similar results have been found for petunia through the study of lines mutant for genes that regulate the production of color, either in the whole flower or in parts of the flower. In lines mutant for genes *An1*, *An2*, and *An11*, the EBGs CHS and CHI are still expressed (Beld et al., 1989; Quattrocchio et al., 1993; Weiss et al., 1993). The gene for F3H is also expressed normally, but the expression of genes encoding DFR and AS is reduced or eliminated (Beld et al., 1989; Weiss et al., 1993). Therefore, in petunia, there is also a regulatory division between EBGs and LBGs, although, unlike in Antirrhinum, this division occurs after F3H, not before it.

A similar separation in transcriptional control is suggested in other species in which the individual genes have not been analyzed as extensively. For example, the flower tube in many tobacco varieties is colorless despite detectable expression of the CHS gene (Fritze et al., 1991; Drews et al., 1992), implying that it is nonexpression of other biosynthetic steps that limits tube pigmentation. It is also interesting to note that where the control of pigmentation has been examined in maize seedlings, as distinct from aleurone, CHS gene expression is induced independently of *R*, although the *A1* gene (an LBG) is activated by *R* (Taylor and Briggs, 1990).

Therefore, in flowers, there appears to be a common regulation of the LBGs such that a set of genes is induced coordinately

by a single mechanism. However, the earlier biosynthetic genes require different regulators, indicating that control of anthocyanin production in petals must be complex.

## SPATIAL CONTROL OF PETAL PIGMENTATION

Within parts of the petals that appear uniformly pigmented, some petal cells may express pigment biosynthetic genes more highly than other cells. For example, in standard Antirrhinum lines, which are self-colored (referred to as wild-type lines; Figure 2A), anthocyanin biosynthesis is restricted to the epidermal cells, as illustrated in Figure 3E, as is expression of all biosynthetic genes. The expression of each biosynthetic gene varies across these flowers; it is highest in the cells of the inner epidermis of the lobes and the cells of both inner and outer epidermis at the base of the flower tube. Gene expression is lowest in the upper region of the tube, which may be developmentally distinct from the base of the tube (Figure 3B; Jackson et al., 1992). This pattern is exactly the same for each biosynthetic gene, to the point that in regions in which the levels of gene expression change significantly across a small number of cells (for example, in the hinge region of the Antirrhinum flower), individual cells will show the same relative expression of EBGs and LBGs (Figures 3C and 3D). Therefore, there can be tight spatial coordination of biosynthetic gene expression even though separate mechanisms regulate their transcription.

The tight association between the expression patterns of different genes implies that there must be some spatial coordination of the different mechanisms regulating biosynthetic gene expression. Such coordination could involve a single transcription factor required for the activation of all biosynthetic genes and operating at limiting levels of activity in particular areas of the flower. Such a transcription factor could interact with several other transcription factors, the combination depending on the particular biosynthetic gene being activated. Alternatively, different regulatory mechanisms could control the expression of different biosynthetic genes, with the activity of each mechanism being controlled by a common spatial signal.

## GENES AFFECTING THE PATTERN OF PETAL PIGMENTATION

The situation described above refers to cellular expression of the biosynthetic genes in uniformly pigmented petal tissue. However, within the petal, production of pigment may be patterned. Some patterns are seen in natural isolates, and other patterns arise through mutation.

Patterns may arise by loss of function of a regulatory gene that has a pattern to its area of activity within the flower. In this case, pigmentation will be lost in those areas with an absolute requirement for the transcription factor but not in those



**Figure 2.** Variation in Pattern and Intensity of Anthocyanin Production in *Antirrhinum* Flowers.

(A) Wild-type *Antirrhinum* flower.

(B) *delila* mutant, showing lack of pigment in tube.

(C) *Eluta* mutant, showing reduction of pigmentation, especially on the outer edges of the lobes and in the upper tube.

(D) *Eluta, delila* double mutant, showing synergistic interaction reducing pigmentation in the lobes, further than *Eluta* alone.

(E) *rosea<sup>colorata</sup>*, showing pigmentation of the inner epidermis of the lobes and in a ring on the tube.

(F) Style, stamen, and calyx of *rosea<sup>colorata</sup>*, showing lack of pigment in other plant parts with this allele.

(G) *rosea<sup>dorsea</sup>*, showing pigmentation of the outer epidermis of the lobes.

(H) Style, stamen, and calyx of *rosea<sup>dorsea</sup>*, showing pigmentation of the other plant parts with this allele.

(I) Wild-type *Antirrhinum* line showing full self-colored pigmentation, for comparison to (J) through (P). This line is isogenic with those carrying the CHS mutations.

(J) Effect of mutation of the CHS gene caused by imprecise excision of the transposon Tam3, which removed 263 bp of the promoter from –63 to –326 bp. CHS expression is reduced in the lobes and at the base of the tube and is completely eliminated in the upper tube.

(K) Effect of deletion of 587 bp of the CHS promoter from –54 to –641 bp. The phenotypic consequences are the same as loss of –63 to –326 bp.

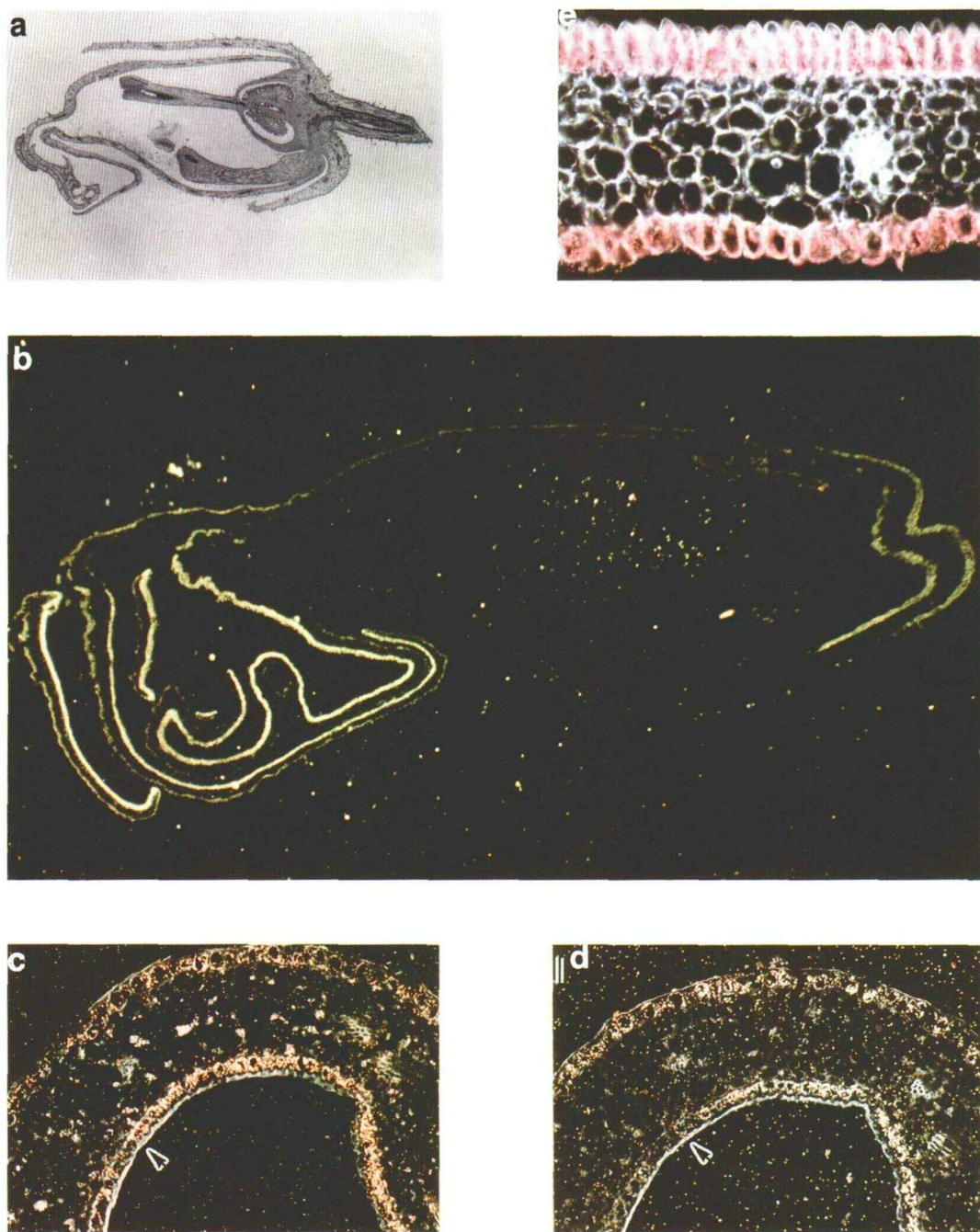
(L) Effect of deletion of 921 bp of the CHS promoter from –50 to –971 bp. The phenotypic consequences are the same as the loss of –63 to –326 bp. These alleles show that *cis*-acting pattern sequences lie between –63 and –326 bp and that no elements further upstream contribute to floral CHS expression.

(M) Effect of inversion that places CHS coding sequences under the influence of a new promoter, which directs gene expression in the upper tube (arrow) but only on the adaxial side. This CHS gene expression is sensitive to the form of the petals because it is lost if the back petals are not formed, as seen in (N).

(N) The *niv* inversion (M) in combination with *cycloidea<sup>adialis</sup>*, in which the back petals do not form. No CHS expression occurs in the upper tube, as indicated by the arrow.

(O) Effect of a *trans*-acting CHS allele in the homozygous form, showing that inverted duplications within the gene proximal region of the CHS promoter almost completely prevent CHS expression in flowers.

(P) Effect of the *trans*-acting CHS allele in combination with a wild-type CHS allele in the heterozygote, showing reduced pigmentation as a result of reduced CHS gene expression, especially at the edges of the lobes.



**Figure 3.** Expression of Anthocyanin Biosynthetic Genes in Developing Antirrhinum Flowers.

(A) Section of flower bud for orientation of in situ.

(B) Expression of CHS transcript across a developing flower bud. Expression is restricted to the epidermis. It is highest in the inner epidermis of the lobes and in both the inner and outer epidermis at the base of the tube. There is a distinct change between high expression in the lobes and low expression in the upper tube. This pattern of expression across the flower is reiterated for each biosynthetic gene.

(C) CHS transcript levels in the hinge region between lobe and tube. Expression levels change sharply, especially in the inner epidermis, as indicated by the arrowhead.

(D) AS transcript levels in the hinge region between lobe and tube. Expression levels change sharply and correspond precisely to the expression levels of CHS (arrowhead).

(E) Section of petal tissue from Antirrhinum showing the epidermal localization of anthocyanin.

areas in which the regulator is not active or in which it can be substituted for by another factor.

A pattern mutation may also arise in a biosynthetic gene itself if the mutation is in a regulatory region of the gene (for example, the promoter) such that it interferes with the interaction between a regulatory gene product and the biosynthetic gene (for example, by modification of a *cis*-acting protein binding motif in a biosynthetic gene promoter; Coen et al., 1986; Almeida et al., 1989). In this respect, it is interesting to note that pattern mutants are common in the CHS (*Nivea*) and DFR (*Pallida*) genes expressed in *Antirrhinum* flowers but are very uncommon in the CHS (*C2*) and DFR (*A1*) genes expressed in maize aleurone, despite the presence of mutagenic transposon insertions in all of these genes (O'Reilly et al., 1985; Coen et al., 1986; Wienand et al., 1986; Sommer et al., 1988). This difference again emphasizes the complex control of biosynthetic gene expression with respect to the spatial pattern in dicot flowers compared to the more uniform spatial control in maize aleurone.

## PATTERNS OF PIGMENTATION IN PETALS

Some patterns of pigmentation follow structural features such as the lobe and tube regions in *Antirrhinum* and the limb and tube zones in tobacco. The epidermal cells in these regions are structurally quite distinct, and the division between regions is marked by a change in the orientation of the petal (Drews et al., 1992).

Other patterns do not follow clear structural boundaries. For example, many wild *Antirrhinum* species have pigmentation concentrated in the face of the lobes, on the inner edge of the two back petals, and at the base of the tube. In these examples, the boundaries between pigmented and unpigmented cells are often rather blurred, and they do not follow clear structural boundaries (Stubbe, 1966). Another well-known example is the "Red Star" phenotype of *petunia* (Figure 1H), in which the division between the pigmented cells and the unpigmented cells of the "star" in the limb is quite distinct but does not follow structural divisions. In addition, the star pattern is variable and depends on environmental conditions (especially light) that do not affect flower morphology.

## HOW ARE PATTERNS OF PIGMENTATION DETERMINED WITHIN THE PETALS?

One gene that contributes to the pattern of flower pigmentation in *Antirrhinum* is *Del*. A functional *Del* product gives a self-colored flower in the absence of other modifiers. In *del* mutants, the flower tube is unpigmented due to lack of expression of the LBGs (Figure 2B). The flower lobes are fully pigmented, and LBG expression in lobes of *del* mutants is normal. *Del* has been cloned and shown to encode a protein very

similar to the protein encoded by the *R* gene family of maize (Goodrich et al., 1992). The *del* product is very likely, therefore, to function as a transcription factor of the basic helix-loop-helix type (bHLH), transactivating the LBGs in flower tubes. It appears to interact with the promoters of the LBGs because inversion of the DFR (*Pallida*) gene promoter upstream of -70 bp releases this gene from control by *Del* in tubes (Almeida et al., 1989; Goodrich et al., 1992).

*Del* is also expressed in flower lobes, but because there is no reduction in LBG expression in *del* lobes, it has been suggested that another factor, with higher affinity for the LBG promoters, transactivates the LBGs in lobes in preference to *Del*. Mutations of the DFR gene promoter that presumably prevent the lobe factor from activating DFR transcription place DFR under the control of *Del* in the lobes, showing that *Del* can substitute for the lobe factor (Almeida et al., 1989; Goodrich et al., 1992). The idea therefore remains that patterns of regulatory gene expression determine patterns of pigment production in petals in the same way as in the maize plant (Ludwig and Wessler, 1990). However, combinations of transcription factors must be involved. The mechanisms that, in turn, control the patterns of expression of the regulatory transcription factors themselves have not yet been identified.

Although *Del* is not required for EBG activation in flower tubes, the absence of the *Del* product in lobes gives rise to ectopic expression of the CHS gene in petal tissue, extending its expression to the mesophyll (Jackson et al., 1992). Therefore, although *Del* is an activator of the LBGs in the flower tube, it is a repressor of the CHS gene in the mesophyll of the lobes. A protein, such as the *Del* product, that has the structure of a transcriptional activator might also be able to repress gene expression if the target gene has no binding site for it within its promoter. In lobes, the *Del* product might interact with other transcription factors that have the potential to transactivate the CHS gene. In binding to these factors, the *Del* product would compete for them with the target gene promoter. Appreciable factor interaction of this type with *Del* could thereby reduce CHS expression.

The homology between *Del* from *Antirrhinum* and the *R* gene family from maize suggests that this type of transcription factor may be in general use in regulating anthocyanin biosynthesis in different species and in different parts of the plant. This view is supported by experiments in which the *R* family member *Lc* has been expressed under the control of the cauliflower mosaic virus 35S promoter in transgenic tobacco and *Arabidopsis* (Lloyd et al., 1992). Both the transgenic tobacco flowers and the transgenic *Arabidopsis* plants are more deeply pigmented than controls. This indicates that the bHLH type of transcription factor may limit the rate of biosynthetic gene expression and anthocyanin production in many species (Goodrich et al., 1992). However, *Lc* does not produce pigment in all transgenic plant tissues, indicating that it is not a master gene for pigment production. It may function by transactivating all the biosynthetic genes, but because CHS is already expressed in tissues showing major increases in pigment, such as tobacco flower tubes, it may only need to activate the LBGs

to increase anthocyanin production in these transgenic systems.

The other gene family regulating anthocyanin biosynthesis in maize is the *C1* family, which consists of transcription factors related to the mammalian proto-oncogene *c-myc* (Paz-Ares et al., 1987). No *myb*-related gene has yet been demonstrated to regulate biosynthetic gene expression in dicot flowers, although overexpression of *C1* in Arabidopsis plants that are also overexpressing a maize *Lc* gene increases the degree of plant pigmentation, especially in roots and flowers (Lloyd et al., 1992). Alone, *C1* does not enhance pigment production. These results do not permit a clear conclusion as to whether a *C1* homolog normally operates in controlling Arabidopsis pigmentation. The synergistic interaction of maize *Lc* and *C1* in transgenic Arabidopsis could result either from maize *Lc* being able to function more efficiently with maize *C1* than with an Arabidopsis *C1* homolog or from the fact that *R* homologs normally work alone in dicots but the maize gene works more efficiently with its regulatory partner. A number of *myb*-related transcription factor genes have been isolated from plants, but whether they function in regulating floral pigmentation is not yet known.

Other genes are known to modify the pattern of pigment within petals, and there is evidence that these genes also influence biosynthetic gene transcription. For example, the semidominant *Eluta* mutation in Antirrhinum restricts pigment to localized areas of the flower face and the base of the tube (Figure 2C). It does this by decreasing the steady state levels of LBG transcripts in both lobes and tubes, but it does not reduce the expression of CHS and CHI genes (Martin et al., 1991). In fact, *Eluta* increases the levels of CHI transcript detected in the flower lobes, implying that, although it may repress the expression of the LBGs, it may also activate CHI gene expression (Martin et al., 1991). *Eluta* lines carrying a nonfunctional *del* gene have less floral pigmentation than lines homozygous for either mutation alone, suggesting that the wild-type *Eluta* and *Del* gene products interact to control LBG expression (Figure 2D).

Although the main influence of *Del* is in the flower tube, it also reduces pigmentation in the lower half of the sepals. In addition, in *del* homozygous mutant seedlings, the hypocotyl is unpigmented, whereas *Del* seedlings have pigmented hypocotyls. Thus, genes regulating floral pigmentation may also have some influence in controlling pigmentation of other plant parts. The pigmentation of stem internodes is not abolished by *del* inactivity, however.

Another gene from Antirrhinum, *Rosea*, has two alleles that reduce floral pigmentation. *rosea<sup>colorata</sup>* reduces pigmentation and restricts it to the inner epidermis of the lobes and a ring in the middle of the tube (Figure 2E). The rest of the plant body is unpigmented (Figure 2F). A second allele, *rosea<sup>dorsea</sup>*, restricts pigmentation to the outer epidermis of the lobes and the same ring in the middle of the tube (Figure 2G). In this case, the rest of the plant body, including the stem and leaves, is pigmented as normal (Figure 2H). Both *rosea* alleles reduce the expression of the LBGs, but they do not affect CHS or CHI gene expression, implying that the *Rosea* product is another

member of a group of transcription factors that regulates the genes encoding the late steps in anthocyanin production in flowers (Bartlett, 1989). The basis for the functional distinction between *rosea* alleles is not yet known.

In petunia, some patterns of coloration result from the specific floral expression patterns of multiple copies of particular biosynthetic genes. For example, the genes *Ht1* and *Ht2* are functionally equivalent, both encoding F3'H, which catalyzes the conversion of dihydrokaempferol to dihydroquercetin. In petunia, DFR shows strong substrate preference for dihydroquercetin over dihydrokaempferol, and little anthocyanin is produced if the activity of F3'H is blocked. *Ht1* is expressed in both limb and tube, whereas *Ht2* is expressed only in the tube (Wiering and de Vlamming, 1984). In plants with no active *Ht1* (*ht1/ht1*) but with active *Ht2*, coloration is dependent on the production of dihydroquercetin and is mainly restricted to the tube but also progresses slightly into the limb area, resulting in a ring of color at the top of the tube (Figure 1F).

Another pattern observed frequently in petunia flowers is dark pigmentation over the veins, with paler pigmentation elsewhere. Loss of function of a single gene, *An12*, may result in this veination pattern (Figure 1E; Gerats et al., 1989). Analysis of unstable mutations of this locus suggests that the gene acts to increase pigment production in the areas beyond the veins, because reversion is from patterned to self-colored flowers. Based on the function of other pigment regulatory genes, *An12* might encode a transcription factor that controls this aspect of pigment production. It seems likely that one of the signals for anthocyanin production is supplied by the vascular system and the *An12* gene promotes pigment production in tissues beyond the reach of this signal or that it facilitates the transport of the signal itself.

Patterns of pigmentation within petals appear, therefore, to result primarily from the differential expression of the pigment biosynthetic genes. This expression is controlled by regulatory genes, which probably encode transcription factors. Some facets of patterning in flower coloration may result from the specific expression patterns of these regulatory genes, although it seems likely that more than two factors coordinate pigment gene expression across the flower and that some individual factors may be able to substitute for others in certain areas of the petals. The regulation of flavonoid production is similar to that in maize in that it is dependent on similar types of regulatory genes, but there are multiple spatial domains for control within the flower, and the genes encoding the enzymes of the biosynthetic pathway are regulated more independently of each other than in maize.

## CONTROL OF CHS GENE EXPRESSION IN FLOWERS

Although the genetic components that regulate the expression of the LBGs in petals are beginning to be characterized, the control of CHS and CHI gene expression in petals remains relatively ill defined. In pea, two genes, *A* and *A2*, are involved

in the control of CHS gene transcription in flowers, although they do not affect CHS gene expression in other parts of the plant (Harker et al., 1990). The molecular basis of this control is not yet understood.

Another approach to understanding the control of CHS gene expression in flowers is to dissect the promoter of the CHS gene to identify regions responsible for expression in petals or in parts of petals. CHS promoter analysis using reporter gene fusions in petunia has indicated that the first 67 bp of the promoter are sufficient for flower-specific expression. Two repeat motifs (TACPyAT) lying between -50 and -62 bp mediate this expression by suppressing expression from the CHS promoter outside flowers (van der Meer et al., 1990, 1992).

TACCAT motifs have also been shown to be of significance in CHS gene expression in *Antirrhinum* flowers (Sommer et al., 1988), although a quantitative rather than a qualitative role has been suggested. Beyond these results, CHS promoter motifs have not been examined with particular reference to the control of expression in flowers, although an enhancer motif lying between -550 and -660 bp in the *Antirrhinum* CHS promoter does not appear to function in petals (Figures 2I to 2L; Staiger et al., 1990; Fritze et al., 1991).

Mutagenesis in vivo of the *Antirrhinum* CHS gene promoter by the transposon Tam3 has given rise to a large number of alleles. Deletion of promoter sequences between -63 and -326 bp or beyond gives patterned pigmentation of flowers, reflecting a new pattern of CHS gene expression (Figures 2I to 2L). Expression is reduced by ~10-fold in the flower lobes and at the base of the tube and is completely abolished in the upper part of the tube. This result shows that factors required for high CHS gene expression and expression in the upper part of the tube interact with promoter sequences between -63 and -326 bp. Interestingly, these sequences include the G-box, which is required for light induction of CHS expression (Schulz-Lefert et al., 1989; Staiger et al., 1989). Light could serve a rather specific function in CHS expression in *Antirrhinum* flowers, enhancing it in the flower generally and inducing it in the upper part of the flower tube through the bZIP family of transcription factors, which are known to bind the G-box (Weisshaar et al., 1991). The differential pigmentation of the upper and lower tube in these CHS gene mutants might also reflect aspects of the separate ontogeny of the upper and lower tube.

Another Tam3-induced rearrangement of the *Antirrhinum* CHS promoter involves an inversion that creates a novel chimeric CHS gene promoter (Figure 2M). In this example, the loss of sequences -63 to -326 bp is partially supplemented by the new sequences, which enhance CHS gene expression slightly in the lobes and which induce CHS gene expression on the adaxial side of the upper tube but not on the abaxial side (arrow in Figure 2M). This novel control appears to operate directly or indirectly through the *cycloidea<sup>radialis</sup>* gene, which determines, inter alia, the morphogenetic differentiation of back petals from lower petals to establish bilateral symmetry, because the combination of the *niv* inversion with a *cycloidea<sup>radialis</sup>* mutation abolishes adaxial tube pigmentation

(Figure 2N). The novel patterns of CHS gene expression caused by the chimeric promoter show that the pattern of CHS gene expression can be established in response to morphogenetic determinants even though the tissues of the fused back and lower petals of the tube are structurally indistinguishable at the time of pigment formation. In this context, it is also interesting to note that CHS gene expression is localized in the upper part of the developing corolla in tobacco before the structural differentiation of tube and limb is fully evident (Drews et al., 1992). Later, CHS expression is concentrated in the limb. It would appear, therefore, that biosynthetic gene expression can respond to morphogenetic determinants both before and after the structural changes they determine are apparent.

Some *trans*-acting alleles of the CHS gene have been described in *Antirrhinum* (Carpenter et al., 1987; Coen and Carpenter, 1988; Martin et al., 1988; Bollmann et al., 1991). These alleles, which themselves give very little CHS expression, reduce the expression from a wild-type CHS allele in the heterozygote. The reduced pigmentation in heterozygotes is patterned within the *Antirrhinum* flower, being greatest toward the outer edges of the lobes and least on the face of the flower (Figures 2O and 2P). Various theories have been advanced to explain this strange effect on the intensity and pattern of pigmentation (Coen and Carpenter, 1988; Bollmann et al., 1991). The most probable explanation is that the alleles contain a binding site with a higher-than-normal affinity for a transcription factor required to activate CHS gene expression, especially at the margins of the flower lobes. The *trans*-acting alleles that have been examined at the molecular level all contain inverted duplications of the CHS promoter region around the -63 position. Such palindromes might have high enough binding affinity to compete successfully for a transcription factor with a single binding motif in the wild-type CHS gene.

## ROLE OF HOMEOTIC GENES IN REGULATING PETAL PIGMENTATION

We have considered the expression of pigment biosynthetic genes against a backdrop of petal morphogenesis, and we have suggested that there may be links between pigmentation patterns and structural determination. It is therefore possible that the homeotic genes that determine floral whorl identity may also play a subsequent role in tissue-specific gene expression because their expression continues late into organ morphogenesis. If this is the case, genes such as *deficiens* (*Def*) and *globosa* (*Glo*) in *Antirrhinum* and *green petals* in petunia might be directly involved in the activation of pigment biosynthetic genes (Sommer et al., 1990; Angenent et al., 1992; Schwarz-Sommer et al., 1992). Although the discovery of specialized regulators such as *Del* argues against such a direct role for homeotic genes, there is some evidence from analysis of revertant somatic sectors of *Def*, seen as small islands of petal cells in sepal tissue, to suggest that *Def* remains functionally active late into organ development and may induce

pigmented petal cell formation late in organ differentiation (Coen and Carpenter, 1992).

In *Antirrhinum*, the homeotic genes (*Def* and *Glo*) required for the *b* function, which determines petal and stamen identity (Coen and Meyerowitz, 1991), encode MADS box transcription factors with the common binding motif CC(AT)<sub>6</sub>GG (Schwarz-Sommer et al., 1992; see also Coen and Carpenter, 1993, this issue). A good approximation of this motif is found in the *Antirrhinum* CHS (*Niv*) gene promoter, and weaker approximations are found in the promoter regions of other biosynthetic genes (Martin et al., 1991). However, there is no strong evidence for direct transcriptional control of the anthocyanin biosynthetic genes by homeotic genes *in vivo*. It seems more likely that *Def* and *Glo* activate genes such as *Del*, which in turn activate parts of the biosynthetic pathway.

### ACTION OF GROWTH REGULATORS DURING PETAL DEVELOPMENT

There is evidence that plant growth regulators induce anthocyanin production during petal development. In emasculated petunia flowers or in isolated petunia petals bathed in sucrose solution, exogenously supplied gibberellic acid (GA<sub>3</sub>) will induce anthocyanin production, which otherwise does not occur (Weiss et al., 1990, 1992). Gibberellins are thought to be synthesized normally by the developing anthers and, in turn, to induce the expression of the anthocyanin biosynthetic genes in the petals to produce color. Exogenous GA<sub>3</sub> induces the transcription of EBGs such as CHS and CHI and LBGs such as DFR and AS (Weiss et al., 1992, 1993). This type of mechanism may represent a method for coordination of anther development and color signal formation. Perhaps gibberellins induce the activity of transcriptional activators of the bHLH or *myb* type to induce color formation. It is not clear how universal the influence of gibberellin is on floral pigmentation. Unfortunately, species such as *Arabidopsis*, for which well-characterized mutants deficient in gibberellin biosynthesis exist, do not produce anthocyanins in their petals. However, a number of dwarf pea varieties exist with impaired gibberellin production (Stoddart, 1987), and these can have colored flowers (Mendel, 1865). Either gibberellin production in pea anthers is determined by different genes from those that act in internodes, or gibberellins are not required for floral pigmentation in pea.

### NONTRANSCRIPTIONAL CONTROL OF FLORAL PIGMENTATION

Not all control of floral pigmentation need be transcriptional control of biosynthetic gene expression. An example of this comes from an analysis of the "Red Star" phenotype of petunia. This phenotype can be centrifugal (starting at the center

of the flower and increasing toward the margin) or centripetal, as shown in Figure 1H. Its genetic determination depends on at least four recessive characters (Levan, 1939) and one dominant one (T. Gerats, unpublished results). Low temperature and high light lead to larger uncolored areas. Surprisingly, the period during which pattern formation can be influenced is very short and takes place early in flower development (between 10 and 15 days before maturation, when flowers are between 1 and 3 mm long; Marheineke, 1936).

It has been shown that the absence of color in the star is associated with an absence of CHS transcript in this region (Mol et al., 1983). However, run-on transcription studies show that the CHS gene is transcribed but that its transcript does not accumulate in the acyanic areas, implying that the star pattern is the result of post-transcriptional control of CHS expression (van der Meer, 1991). In this respect, the early influence of environment is fascinating, because the temperature- and light-sensitive period comes long before the biosynthetic genes are transcribed. Treatment with GA<sub>3</sub> can completely suppress the formation of the star pattern, whereas treatment with 2,2-dimethyl hydrazide (B9), a growth retardant, can lead to fully white flowers (van der Krol et al., 1989). The combination of these results suggests that the mechanism determining the "Red Star" phenotype could be preprogrammed considerably before it is actually operational. Gibberellins may be involved in this control, implying that these growth regulators may influence flavonoid production both transcriptionally and through post-transcriptional control of gene expression.

### CONCLUSION

Color production is an integral part of the development of many flowers and is essential to their successful functioning in sexual reproduction. The control of color production in petals appears to be vested primarily in transcriptional control of expression of the anthocyanin biosynthetic genes. This control is complex; different regulators control different parts of the biosynthetic pathway, and several transcription factors are involved. There is a multitude of different coloration patterns in flowers, and evidence to link aspects of these patterns to morphogenetic determination is beginning to emerge. Other signals, including growth regulators and light, are also involved in regulating floral pigmentation. Understanding the mechanistic links between the genes determining floral morphogenesis and the genes controlling pigmentation will throw further light on the development of pigment patterns in petals.

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